Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of *Plasmodium falciparum*

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The antimicrobial biocide triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol] potently inhibits the growth of *Plasmodium falciparum in vitro* and, in a mouse model, *Plasmodium berghei in vivo*. Inhibition of [^{14}C]acetate and [^{14}C]malonyl-CoA incorporation into fatty acids *in vivo* and *in vitro*, respectively, by triclosan implicate FabI as its target. Here we demonstrate that the enoyl-ACP reductase purified from *P. falciparum* is triclosan sensitive. Also, we present the evidence for the existence of *FabI* gene in *P. falciparum*. We establish the existence of the *de novo* fatty acid biosynthetic pathway in this parasite, and identify a key enzyme of this pathway for the development of new antimalarials.

The enzymes involved in the biosynthesis of fatty acids are organized in two distinct ways within living systems[1]. Fungi, mammals and some mycobacteria accomplish fatty acid synthesis by multifunctional proteins in which each reaction is catalyzed by a distinct region (domain) of these large proteins. These enzymes are classified as type I fatty acid synthases and are also described as the 'associative' type of fatty acid synthases, because successive steps in the fatty acid synthetic reaction occur at specific domains[1]. Plants and many bacteria, however, use the type II or 'dissociated' fatty acid synthases that are best characterized in *Escherichia coli*[2].

Each of the individual reactions in fatty acid synthesis in *E. coli* and plants is carried out by separate enzymes in contrast to the single multifunctional enzyme of the mammalian system. Acetyl-CoA and malonyl-acyl-carrier protein (malonyl-ACP) or acetyl-ACP and malonyl-ACP react to form acetoacetyl-ACP in a condensation reaction catalyzed by the β-ketoacyl-ACP synthase III (FabH) or acyl-malonyl-ACP condensing enzyme (FabB or FabF), respectively. The condensation reaction is followed by a reduction, a dehydration and a second reduction catalyzed by β-ketoacyl-ACP reductase (FabG), β-hydroxyacyl-ACP dehydrase (FabA or FabZ) and enoyl-ACP reductase (FabI)[2-4], respectively. These in turn convert acetoacetyl-ACP to butyryl-ACP. Palmitic acid formed by replication of this cycle can be either elongated by another set of enzymes or channelized for the formation of phospholipids and other molecules.

Though several enzymes are involved in the condensation and dehydration reactions, the final step of elongation is catalyzed by a single enzyme, enoyl-ACP reductase[4]. Moreover, enoyl-ACP reductase is the rate-controlling element in completing the rounds of fatty acid elongation[5]. The presence of separate nuclear encoded genes for ACP (*FabH*, *FabZ* and *FabF*) that are targeted to apicoplast— a plastid-like organelle implicated in fatty acid synthesis in malaria parasite— indicates the existence of type II fatty acid synthase in this organism[6].

Given the key regulatory role of FabI in the dissociative-type fatty acid synthesis system, we investigated inhibiting this enzyme in the malaria parasite with triclosan, a hydroxydiphenylether that has recently been shown to target enoyl-ACP reductase in bacterial systems[7-9]. The results show inhibition of the growth of *Plasmodium* both *in vitro* as well as *in vivo* at low concentrations. We also show the respective inhibition by triclosan of the incorporation of 1,2[^{14}C]acetate and [^{14}C]malonyl-CoA into fatty acids by *P. falciparum* cultures *in vivo* and in cell-free system. We also purified the enzyme from *P. falciparum* and characterized its enoyl-ACP reductase activity. Triclosan binds to it and inhibits its activity. An ORF in the latest deposition of the *P. falciparum* sequence in the *Plasmodium* database corresponds to *FabI* and we cloned the same (http://www.sanger.ac.uk/; ORF: mal_BU-128e03.plc_1). Together, the fatty acid synthesis in *P. falciparum*, the occurrence of FabI and the antimalarial activity of triclosan are important for the development of new chemotherapeutic agents targeting fatty acid synthesis for the treatment of malaria—a disease acquiring alarming proportions due to the resurgence of drug resistant strains.

Triclosan inhibits *Plasmodium* growth *in vitro*

We quantified antimalarial activity of triclosan and its dose response curve by monitoring [^{3}H]hypoxanthine uptake as an index of growth, where we found 0.7 μM to be 50% inhibitory (Fig. 1*a*). We found another member of this class of compounds, 2,2' dihydroxydiphenyl ether, to be 0.1% as effective an inhibitor of the *in vitro* growth of *P. falciparum* (IC_{50} value, 1 mM; data not shown). Cerulenin, an antibiotic and a non-competitive inhibitor of fatty acid synthase[11], also inhibited *Plasmodium* growth (IC_{50} value, 20 μM). The IC_{50} value of triclosan for the resistant strain (MP-14) was almost the same (1.2 μM). Coincubation of both the inhibitors at their respective IC_{50} values almost completely abrogated the growth of the parasite (> 98%; data not shown). We then examined the ability of triclosan to arrest growth of malaria parasites in a stage-specific manner. Merozoites and rings were refractory to the inhibitory effects of the drug, whereas young

trophozoites were highly susceptible (Fig. 1*c* and *d*). These trophozoites did not invade fresh red blood cells (RBCs) after 48 hours as seen in control (Fig. 1*b*).

Triclosan inhibits *Plasmodium* growth *in vivo*

We next examined the efficacy of antimalarial activity of triclosan *in vivo* in *Plasmodium berghei*. Single subcutaneous injection of triclosan (3.0 mg/kg) inhibited 75% parasitemia within 24 hours of drug administration, whereas 38 mg/kg completely cleared the parasite from circulation with one injection (Table 1). Moreover, whereas all the untreated control mice ($n = 6$) developed more than 70% parasitemia on day 5 and died by day 8, mice receiving 38 mg/kg of triclosan, survived for several weeks (> 6; Table 1). Together, these data confirm the efficacy of triclosan in inhibiting the growth of malaria parasite both *in vitro* and *in vivo*. We observed no side effects of triclosan at 40 mg/kg as monitored by checking the activities of the enzymes serum glutamate-pyruvate transaminase (SGPT) and serum glutamate-oxaloacetate transaminase (SGOT) as well as urea, glucose and creatinine levels in the serum. The levels of SGPT, SGOT, urea, glucose and creatinine were 13.2 U/l, 33.2 U/l, 20.7 mg/dl, 97.2 mg/dl and 0.81 mg/dl, respectively, before administration of triclosan. These parameters were also measured subsequently on day 8 and 15 of the treatment. The parameters for SGPT, SGOT, urea, glucose and creatinine on day 8 and 15 were, respectively, 12.6 & 13.7 U/l, 37.2 & 32.4 U/l, 18.3 & 19.8 mg/dl, 93.0 & 88.2 mg/dl and 0.78 & 0.74 mg/dl (ref. 11).

Table 1 *In vivo* antimalarial activity of triclosan*

Dosage (mg/kg)	Parasitemia after 4 d of injections of triclosan (%)
38	0.0
28	5 ± 7
14	10 ± 9
8	10 ± 10
3	20 ± 15
1.6	40 ± 5
0.8	65 ± 20
control	68 ± 18

Mean values ± s.d. from 3 to 4 independent experiments, each containing five to six mice per dosage, are shown. *In another set of experiments 40 mg/kg triclosan was injected SC (day 2 of infection) when mice had a high level of parasitemia (20 ± 7%) for 4 d. All 4 mice in the treated group showed complete clearance of the parasite by day 8, post-infection, and survived for 6 wk whereas the untreated mice died by day 8.

Triclosan acts by inhibition of parasite fatty acid synthesis

We initially investigated the ability of the parasite to synthesize fatty acids. Contrary to earlier reports[12–14], we saw incorporation of not only 1,2[^{14}C]acetate but also [^{14}C]malonyl-CoA into fatty acids. Mostly we detected the synthesis of fatty acids of chain lengths C-10, C-12 and C-14 (Fig. 2). The profile of the fatty acids synthesized by *P. falciparum* is different from that observed for bacteria. In *Salmonella typhimurium* and *E. coli*, in contrast to the malaria parasite, the synthesis of C-14 and C-16 fatty acids is predominant[3]. If FabI activity was inhibited by triclosan, the incorporation of 1,2[^{14}C]acetate—used here as the index for the fatty acid synthesis in *P. falciparum*—should be reduced, and 2μM triclosan inhibited this incorporation by 50% (Fig. 2*a* and *b*). Moreover, triclosan inhibited the incorporation of the [^{14}C]malonyl-CoA in the cell-free fatty acid synthesizing system (Table 2). Cerulenin inhibited [^{14}C]malonyl-CoA incorporation into fatty acids and acted synergistically with triclosan (Table 2).

Plasmodium ACP reductase is NADH dependent

Enoyl-ACP reductase activity with partially purified extracts showed a greater dependency of the protein on NADH than NADPH (Table 2). Also, at high concentrations, NADH in the assay system can arrest the inhibitory effect of triclosan (Table 2). Chloroquine, at 100 μM, had no effect on fatty acid synthesis (Table 2).

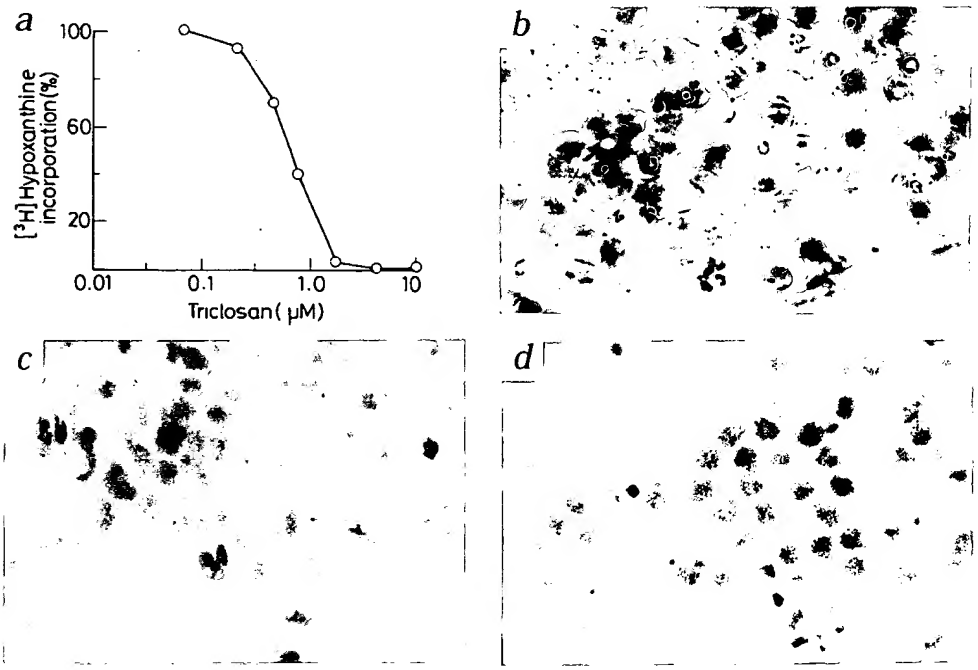


Fig. 1 *In vitro* antimalarial activity of triclosan. ***a***, [^{3}H]hypoxanthine uptake by the parasite. ***b-d***, Giemsa stained smears of triclosan untreated/treated parasites. *b*, control treated 48 h. c, Triclosan treated 24 h. *d*, Triclosan treated 48 h.

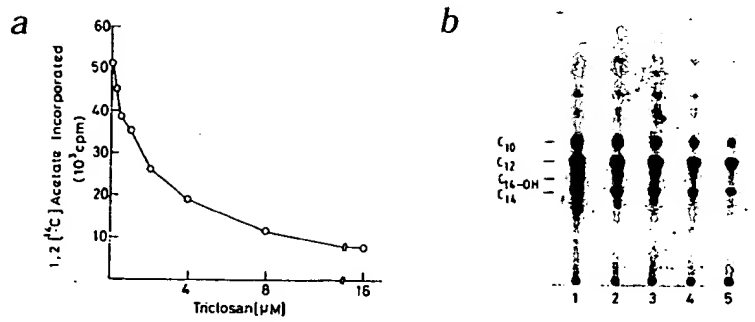


Fig. 2 Inhibition of fatty acid synthesis by triclosan. *a*, *In situ* incorporation of 1,2[^{14}C]acetate into fatty acids by *P. falciparum*. *b*, TLC pattern of fatty acids synthesized and their inhibition by triclosan. Arrows indicate the position of the standard fatty acid methyl esters. Horizontal line at the top of lane 1 marks the solvent front. Lanes 1–5 contained 0, 0.25, 0.5, 1 and 2 μM of triclosan, respectively, during 1,2[^{14}C]acetate incorporation.

Plasmodium FabI protein is a 34 kD protein

ACP affinity-purified enzyme (21 μg) had specific activity of 180–220 U/mg. On 12% SDS-PAGE it showed a band of approximately 34 kD (Fig. 3*a*). It had Glu-Lys-Glu-Glu-Gln-Asp-Ala as its amino-terminal sequence, which is similar to the N terminus of the mature enzyme from *Brassica napus*[16]. The enzyme used crotonyl-CoA and crotonyl-ACP as substrates (Fig. 3*b*). Triclosan inhibited this reaction as shown by the diminution in the crotonyl-ACP-dependent oxidation of NADH monitored by decrease in the absorbance of the latter at 340 nm (Fig. 3*b* and *c*).

Triclosan promotes the association of NAD$^+$

Triclosan promotes the binding of the oxidized cofactor to enoyl-ACP reductase, which is hardly seen in its absence (Fig. 3*d*). 3 μM triclosan is sufficient for 50% saturation of NAD$^+$ binding to the *P. falciparum* enzyme (Fig. 3*d*).

FabI is present in *Plasmodium*

Reverse trasnscriptase (RT)-PCR, using primers for *Plasmodium FabI* gene sequence present in database showed an expected 1.3 kilobase band (Fig. 4*a*). The cloned gene from the PCR product gave the same sequence as reported in the database. *Plasmodium* enoyl-ACP reductase has 37.4% and 42.2% identity with *B. napus* and *Chlamydophila pneumoniae* enzymes, respectively[16] (Fig. 4*b*). Whereas Y156, M159 and F203 implicated in triclosan binding in the *E. coli* FabI (ref. 17) are conserved in the plasmodial enzyme (that is, Y281, M285 and F372), G93 of the former is replaced with an alanine (A217). Replacement of the glycine with an alanine in the parasite enzyme is perhaps tolerated because of its location in a relatively shorter loop as compared with that of the bacterial protein.

Discussion

The apicoplast[18], an organelle of endosymbiotic, eukaryotic algal origin, is indispensible for *P. falciparum* and is implicated in harbouring the enzymes of fatty acid synthesis. This led us to examine the existence of this important biosynthetic pathway in the parasite as well as its disruption by triclosan, a biocide that inhibits fatty acid synthesis in bacteria by specifically targeting FabI, an essential enzyme of this pathway[6–8].

Despite the previous inability to detect incorporation of acetate into fatty acids[12–14], we demonstrate the existence of fatty acid synthesis in this parasite. We attribute earlier unsuccessful attempts to incorporate [^{14}C]acetate to the usage of small amounts of [^{14}C]acetate, unsynchronized cultures with low parasitemia and inappropriate incubation periods. A distinct pattern of fatty acid synthesis in *P. falciparum* sets it apart from other bacterial systems[3] (Fig. 2*b*). Though our cultures were free of any mycoplasma contamination, their contribution to any of the observations reported here can be dismissed by the fact that mycoplasma lack the *de novo* fatty acid synthesis and survive by their uptake from the medium[19–21]. Moreover, *P. falciparum* cultures freed of mycoplasma by treatment with the mycoplasma removal agent (MRA) and then cultured

Table 2 Incorporation of [^{14}C]malonyl-CoA by the cell-free fatty acid synthesis system prepared from *P. falciparum*.

NADH (mM)	NADPH (mM)	NAD (mM)	Triclosan (μM)	Cerulenin (μM)	Chloroquine (μM)	Incorporation of [^{14}C]-malonyl-CoA into fatty acids (c.p.m.)
-	-	-	-	-	-	5,400
-	0.10	-	-	-	-	21,400
0.10	-	-	-	-	-	93,300
0.10	0.10	-	-	-	-	110,300
0.10	-	-	1.0	-	-	78,500
0.10	-	-	2.0	-	-	66,300
0.10	-	-	4.0	-	-	50,300
0.10	-	-	8.0	-	-	36,200
0.10	-	-	16.0	-	-	18,500
2.0	-	-	8.0	-	-	57,300
5.0	-	-	8.0	-	-	91,200
0.10	-	1.0	8.0	-	-	8,200
0.10	-	-	-	25.0	-	52,200
0.10	-	-	8.0	25.0	-	16,200
0.10-	-	-	-	-	100	95,600

Incorporation of [^{14}C]malonyl-CoA was carried out with cell-free fatty acid synthesis system (230–250 μg protein) prepared from *P. falciparum* cultures as described in the text. Each assay in addition to the above contained 70 mM sodium phosphate buffer pH 7.0, 1.4 mM dithiothreitol, 0.14 mM EDTA, 3.6 mM glucose-6-phosphate, 1 U glucose-6-phosphate-dehydrogenase, 20 μM acetyl-CoA and 120 μg of ACP. Other components and their concentrations in the assay used are as indicated in the table. Data reported are from an average of 2–3 experiments.

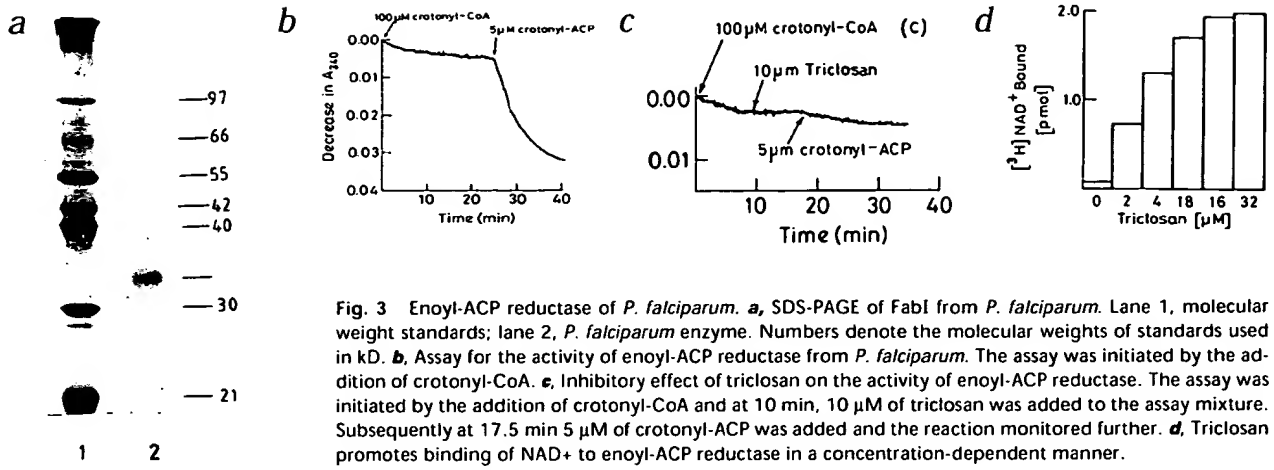


Fig. 3 Enoyl-ACP reductase of *P. falciparum*. ***a***, SDS-PAGE of FabI from *P. falciparum*. Lane 1, molecular weight standards; lane 2, *P. falciparum* enzyme. Numbers denote the molecular weights of standards used in kD. ***b***, Assay for the activity of enoyl-ACP reductase from *P. falciparum*. The assay was initiated by the addition of crotonyl-CoA. ***c***, Inhibitory effect of triclosan on the activity of enoyl-ACP reductase. The assay was initiated by the addition of crotonyl-CoA and at 10 min, 10 μM of triclosan was added to the assay mixture. Subsequently at 17.5 min 5 μM of crotonyl-ACP was added and the reaction monitored further. ***d***, Triclosan promotes binding of NAD+ to enoyl-ACP reductase in a concentration-dependent manner.

again with it, showed the incorporation of [^{14}C]acetate and pattern of fatty acid synthesis similar to that shown by the malaria parasite not treated with MRA.

The incorporation of [^{14}C]malonyl-CoA in a cell-free fatty acid synthesis system further confirms the existence of the *de novo* fatty acid biosynthetic pathway in *P. falciparum*. We also show its inhibition by triclosan and prevention of the same by an excess of the reduced cofactor NADH. Moreover, triclosan used in combination with cerulenin (both used at their IC_{50}) showed a synergy in their action (Table 2). Thus, triclosan inhibits growth of *P. falciparum* by inhibiting *de novo* fatty acid synthesis.

The most important observations are the *in vivo* studies of *P. berghei* in the mouse model. Like chloroquine[22], 3.0 mg/kg triclosan inhibits 75–80% parasitemia with a single subcutaneous injection. Total rescue of mice with high parasitemias (20 ± 7%), post-triclosan treatment, emphasizes the antimalarial efficacy of the drug. Mice treated with triclosan were as healthy and active as their untreated counterparts and had normal parameters for liver and kidney function tests.

The inhibition of fatty acid synthesis by triclosan in the parasite cell-free system pointed to the presence of enoyl-ACP reductase, a key enzyme of the type II fatty acid synthase. Additionally, triclosan seems to be much more powerful than thiolactomycin, another inhibitor of type II fatty acid synthesis[6]. Thiolactomycin inhibition of malaria provided initial evidence for a type II fatty acid biosynthetic pathway in apicomplexan. Our results validate the presence of type II lipid synthesis system in *Plasmodium*. However, no attempt was made in the studies with thiolactomycin to demonstrate the operation of the *de novo* fatty acid synthesis in this apicomplexan parasite[6].

A putative protein with a molecular weight of of 34 ± 2 kD has been purified from *P. falciparum* that exhibited enoyl-ACP reductase activity. This activity was disrupted in a potent manner by triclosan. The N terminus of the purified protein showed similarity with *B. napus* enoyl-ACP reductase[16]. While the manuscript was under revision, we came across an ORF in the *Plasmodium* database, the sequence of which matched a clone for *FabI* derived from *P. falciparum*. Moreover, its N terminus matches with the region downstream of its leader peptide.

Chloroquine has been the most frequently used drug for treating malaria for the last 50 years, but its mode of action and the target continue to be enigmatic, precluding rational design of more potent analogs[23,24]. Among the newly reported drugs that target the parasite, triclosan is clearly more potent[6,18,25]. Triclosan is widely used in the consumer industry ranging from children's toys to toothpastes, detergents and soaps[26]. Discovery of the target for triclosan might allow development of more potent analogs for treating malaria.

In summary, our studies demonstrate the occurrence of *de novo* fatty acid synthesis in *P. falciparum* as well as show the presence of FabI, an essential enzyme of the pathway and identify a novel physiological target for triclosan in malaria parasite that should widen our arsenal for combating malaria.

Methods

Maintenance of cultures. We cultured two strains of *P. falciparum*, FCK2 (CQ-sensitive; IC_{50}, 18 nM) and MP-14 (CQ-resistant; IC_{50}, 300 nM), isolates from Karnataka and Maharashtra states of India, respectively, using standard techniques[27,28] and checked routinely for mycoplasma infection using PCR (ref. 29). Synchronized parasites[30] (12–15% parasitemia) were released from the red blood cells with 0.15% saponin[31].

Growth inhibition assay. We assessed *P. falciparum* growth by measuring the incorporation of [^{3}H]hypoxanthine (ref. 32) with synchronized parasites at ring stage with 1–2% parasitemia. Aliquots of stock solution of triclosan were placed in tissue culture plates (Nunc, Copenhagen, Denmark), to final concentration of 10–0.075 μM in 0.005% DMSO after the addition of uninfected or infected red cells suspension in culture medium. The plates were placed in candle jars and incubated at 37 °C for 4, 28 or 52 h for assessing the growth at 24, 48 and 72 h, respectively. [^{3}H]Hypoxanthine (Amersham; 25.1 Ci/mmol, 5–20 μCi/ml, final concentration) was then added to each well at these time points (5% vol/vol) and after a further 20 h incubation, cells were collected (Skatron cell harvester, Tranky, Norway) and the radioactivity measured by liquid scintillation counting (Wallac 1409, San Francisco, California). Giemsa-stained smears were microscopically examined for parasite morphology before the start of [^{3}H]hypoxanthine uptake and at the end of it.

The *in vivo* antimalarial activity of triclosan in *P. berghei*. We determined the *in vivo* activity of the inhibitor with a modified 4 d suppressive test[33]. BALB/c mice were intravenously inoculated with 1 × 10^7 infected erythrocytes on day 0. On day 1, infection was confirmed and the treatment was started. Various doses of triclosan were administered subcutaneously once a day, over a period of 4 d. Parasitemias were determined every day by Giemsa-stained blood smears for 5 d and thereafter. The survival of the mice was monitored for the next 6 wk. Triclosan (40

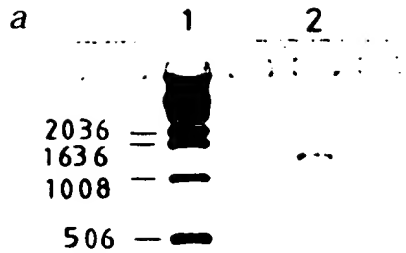
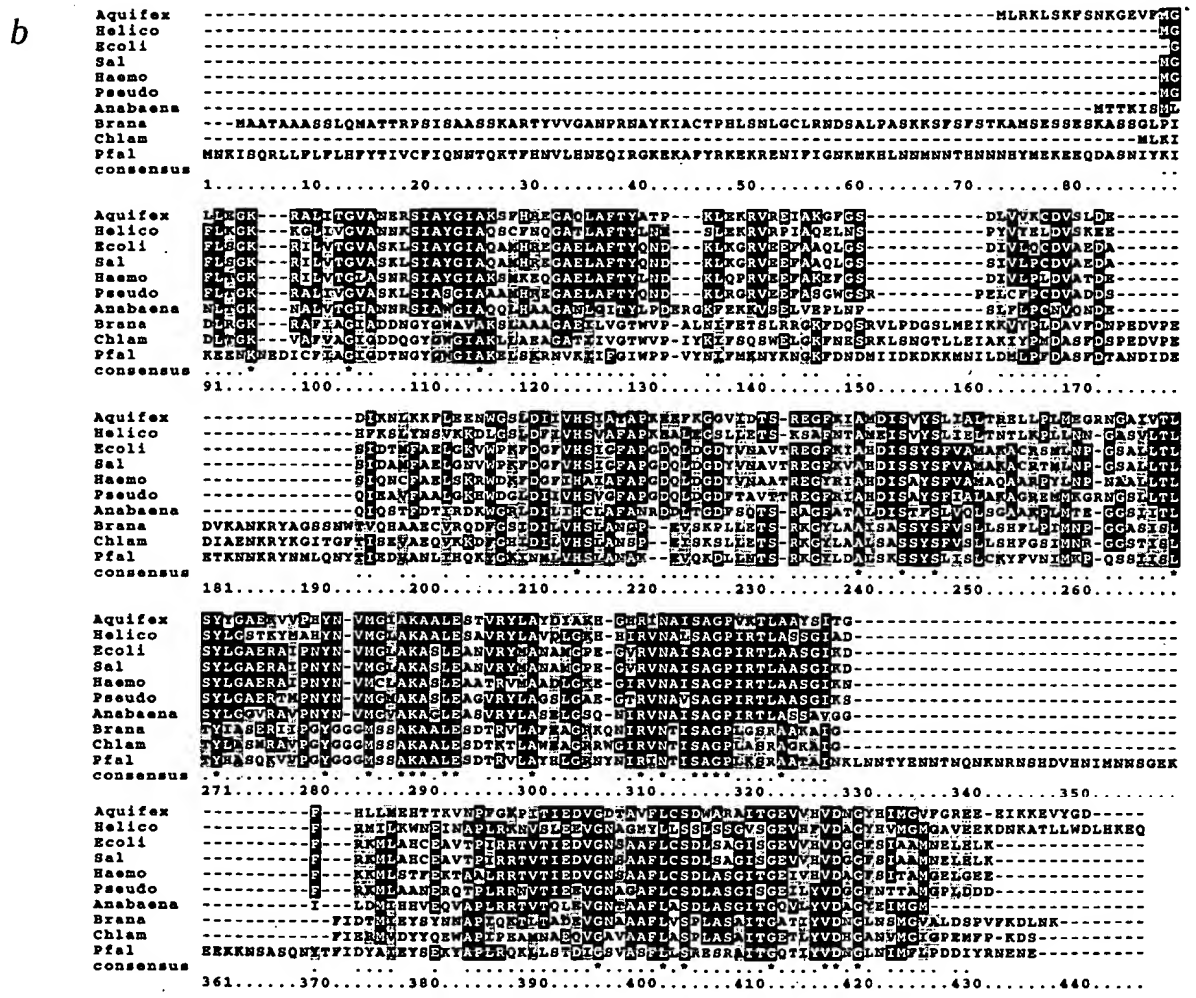


Fig. 4 *FabI* gene of the parasite. ***a***, The RT-PCR products were analyzed on 1% agarose gel. Lane 1, 1 kb DNA ladder, lane 2, 1.3 kb RT-PCR product. ***b***, Alignment of the deduced amino acid sequence of the *P. falciparum* enoyl-ACP reductase with sequences from the homologous enzymes from other organisms. Aquifex, *Aquifex aeolicus* (GenBank accession number gi:2983915); Helico, *Helicobacter pylori* (GenBank accession number gi:2313282); Ecoli, *Escherechia coli* (GenBank accession number gi:1787545, Swiss-Prot accession number P29132); Sal, *Salmonella typhimurium* (GenBank accession number gi:119392, Swiss-Prot accession number P16657); Haemo, *Haemophilus influenzae* (GenBank accession number gi:1574591); Pseudo, *Pseudomonas aeruginosa* (GenBank accession number gi:9947790); Brana, *Brassica napus* (GenBank accession number gi:237650); Chlam, *Chlamydia pneumoniae* (GenBank accession number gi:8978778); Pfal, *Plasmodium falciparum* (The Sanger Centre, http://www.sanger.ac.uk; ORF: mal_BU-128e03.plc_1 on chromosome: BLOB). Black and grey outlines indicate identical and similar amino acid residues, respectively



mg/kg) was also injected daily for 4 d to mice that had developed high levels of parasitemia (20 ± 7%) by day 2 and were monitored as above.

Effect of triclosan on the incorporation of [^{14}C]acetate into fatty acids in *P. falciparum* cultures. We added triclosan (2–16 μM) to 200 ml cultures that were then resuspended in 6.0 ml of the complete medium, while retaining the same concentration of the inhibitor. To this, we added 1,2[^{14}C]acetate (50 μCi/ml, sodium acetate 60 mCi/mmol, NEN). After 2 h, parasites were isolated, washed thoroughly with PBS, lysed, sonicated, spotted onto a Whatman 3 MM paper disc and counted in the scintillation fluid[7]. The incorporation of 1,2[^{14}C]acetate was linear up to 4 h. For the analysis of fatty acids synthesized, and for monitoring the effect of triclosan on the synthesis, we collected a portion of the above cultures (≈ 20 ml) and isolated parasites as described. The parasite pellet was treated with 4M HCl at 100 °C for 2 h. The liberated fatty acids were extracted in chloroform, methylated at 4 °C with diazomethane in ether and chromatographed on silanized silica thin-layer plates[34] (Merck, Damstadt, Germany).

Cell-free fatty acid synthesis by *P. falciparum* extracts. We suspended the trophozoites isolated from 100 ml cultures[31] in 0.2 ml of 70 mM potassium phosphate buffer (pH 7.0), sonicated for 5 s and centrifuged at 48,000 g for 1 h at 4 °C. The supernatant was used as crude extract for determining the *in vitro* fatty acid synthesis[15]. The *in vitro* fatty acid synthesis assay mixture (200 μl) contained: 70 mM potassium phosphate (pH 7.0), 1.4 mM dithiothreitol, 20 μM acetyl-CoA, 3.6 mM glucose 6-phos-

phate, 0.14 mM EDTA, 100 μM of NADH and/or 100 μM of NADPH as indicated, 1 U glucose-6-phosphate dehydrogenase, 230–250 μg of parasite protein and 80 μM [^{14}C]malonyl-CoA (specific activity 54.2 mCi/mmol, NEN). The reaction was initiated by addition of solution containing 120 μg of freshly reduced ACP (ref. 15). The reaction mixture was incubated for 35 min with or without inhibitors (added just before the addition of reduced ACP solution) at 37 °C, and incorporation of [^{14}C]malonyl-CoA into fatty acids was measured by liquid scintillation counting[7].

Purification of enoyl-ACP reductase. We subjected the cell extract (10–12 mg) prepared as above from 2000 ml infected erythrocytes with 12–15% parasitemia to DEAE cellulose, Blue Sepharose 4B and ACP-Sepharose 4B chromatography as described[16,35,36].

Enzyme assay. We assayed enoyl-ACP reductase at 30 °C by monitoring the decrease in absorbance at 340 nm due to the consumption of NADH (ε_{340} = 6220 M^{-1} cm^{-1}). Crotonyl-CoA was used as substrate[35]. The standard reaction mixture in a final volume of 100 μl contained 100 mM sodium phosphate buffer pH 7.5, 100 μM NADH, 2 μg purified enoyl-ACP reductase and 100 μM crotonyl-CoA. We defined one unit of enoyl-ACP reductase activity as the amount required for the oxidation of 1 μmol NADH per min. Protein was quantified as described[37]. Inhibition with triclosan was checked with crotonyl-ACP.

Evaluation of the effect of triclosan on [^{3}H]NAD$^+$ binding. We preincubated affinity-purified *P. falciparum* enoyl-ACP reductase (1.5 μg) with the varying concentrations of triclosan (2–32 μM) for 15 min at 30 °C in 40 μl of 100 mM sodium phosphate buffer (pH 7.5) containing 3% dimethyl sulfoxide. Subsequently, 2.0 μM [^{3}H]NAD$^+$ (specific activity 29 Ci/mmol, NEN) in 5 μl of the above buffer was added and the solution incubated for 25 min. The mixture was then loaded onto 0.45 micron polyvinyldene difluoride membrane, the filters were washed with the buffer and subjected to counting in scintillation fluid[9].

SDS–PAGE and N-terminal sequence analysis. We performed SDS-PAGE on 12% gel in Bio-Rad mini-protean II electrophoresis cell as decribed[38] and electroblotted using 0.8 mA/cm^2 on pre-activated PVDF membranes (Sigma) at 500–550 V for 2 h and the membranes washed with Milli-Q water. The blot was used for sequencing on a Shimadzu automated gas phase sequencer equipped with an online CR4A120A chromatopac phenylthiohydantoin analyzer.

Isolation of RNA. We isolated total RNA from 10 ml packed infected erythrocytes (10–12% parasitemia) after saponin lysis, by the single step method of RNA isolation[39]. The isolated RNA was treated with RQ1 RNase-free DNase (Promega; 1 U/μg RNA) for 45 min at 37 °C and repurified by phenol:chloroform extraction and ethanol precipitation. The RT-PCR was carried out by using one step RT-PCR Kit (Qiagen, Valencia, California). PCR was done with the primers PfFabfor (5′-ATGAATAAAATATCACAACGGTTATTATTCCTC-3′) and PfFabrev (5′-TTCATTTTCATTGCGATATATATCATCTGG-3′). We derived the sequences for these primers from data for *P. falciparum* BLOB, obtained by the blast search of *E. coli FabI* sequence against *P. falciparum* genome.

Cloning and sequencing of RT-PCR product. We excised RT-PCR product from 1% agarose gel and extracted DNA by adsorption onto silica-gel particles (QIAEX II gel extraction system, Qiagen). The PCR product was ligated in pGEM-T vector system (Promega) and transformed in DH5α competent cells. Plasmid DNAs from the white colonies obtained were prepared by SDS/alkali lysis[40]. The recombinant plasmids were sequenced on an ABI Prism model 377 semi-adaptive version 3.0 using T7 promoter.

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